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Melittin-induced changes in thylakoid membranes: particle electrophoresis and light scattering study

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Abstract

Thylakoids were used as a model system to evaluate the effect of bee venom peptide melittin (Mt) on membrane surface charge. At neutral pH, thylakoid membrane surfaces carry excess negative electrical charge. Mt strongly altered the electrophoretic mobility (EPM) of 'low-salt' thylakoids and did not significantly change the EPM of 'high-salt' thylakoids. Mt increased the primary ionic-exchange processes across the 'low-salt' thylakoid membranes, while it did not affect those of 'high-salt' thylakoids. Mt decreased the proton gradient generation on the membranes at both ionic strengths, but it affected more strongly the 'high-salt' than that of 'low-salt' thylakoids. The primary photochemical activity of photosystem II, estimated by the ratio F_v/F_m, was not influenced by the low Mt concentrations. It decreased only when chloroplasts had been incubated with higher Mt concentrations and this effect was better expressed in 'low-salt' than in 'high-salt' thylakoid membranes.

Keywords: Melittin; Thylakoid membranes; Electrophoretic mobility; Surface charge; Light scattering; Chlorophyll fluorescence

1. Introduction

Melittin (Mt), the main component of the European honeybee *Apis mellifera*, is a cationic amphiphilic peptide, which binds to membranes. Mt is a surface-active, amphipathic peptide and serves as a useful model for a variety of membrane interactions [1]. Mt exhibits voltage-dependent channel formation in lipid bilayers [2]. Mt carries highly hydrophilic residues (2 Lys, 2 Arg, 2 Gln at the C-terminus) [3]. Its binding to negatively charged membranes was distinctly enhanced compared to neutral membranes. Because of its amphiphilic properties, it can disrupt lipid bilayers like detergents [4,5]. It was also suggested the possibility of action of Mt on membrane proteins

Abbreviations: EPM, electrophoretic mobility; LS, light scattering; Mt, melittin; PMS, phenazine methosulfate; Chl, chlorophyll; F_0, initial chlorophyll fluorescence; F_v, variable chlorophyll fluorescence; F_m, maximum chlorophyll fluorescence; PS I, photosystem I; PS II, photosystem II.

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through electrostatic forces [6,7]. In solution of low ionic strength the peptide has no regular secondary structure. This peptide is very soluble in water (>250 mg ml^{-1}) [3], but it interacts spontaneously with biological membranes, increasing the bilayer permeability to ions or molecules [8,9]. An effective charge of Mt of approximately 2 was found, which is much lower than the electrical charge of 5–6, which is expected at pH 7.4 [10]. Benachir and Lafleur [11] demonstrated that the protective effect of negatively charged lipids was preserved for asymmetric membranes. It has been reported that both plant chloroplast lipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) in egg phosphatidylcholine vesicles inhibit the lytic activity of Mt [12]. The galactosyldiglycerides MGDG and DGDG are the basic lipid components of the membrane system of higher plant chloroplasts. They are slightly polar uncharged lipids. The negatively charged polar lipids such as phosphatidylglycerol or sulfolipid could also be responsible for the negative surface charges of intact chloroplasts [13]. Berg et al. [14] showed that Mt selectively inhibited the electron transport between the sites of quinone and quinonediimine reduction and may prove useful also as a probe for locating some specific sites within the membrane. Events involved in Mt-induced aggregation of thylakoid membranes and ionic-exchange processes alteration were studied in thylakoids [15,16].

The stacking of chloroplasts membranes is unique for plant cell membranes [17,18]. The mechanism of stacking and separation of photosystem I (PS I) and photosystem II (PS II) in plant thylakoid membranes was widely investigated [19]. When chloroplasts are unstacked in vitro by suspension in low-salt buffers the membranes will possess a net negative charge and the electrostatic field associated with this charge will tend to repel a similar field located nearby and keep the membranes apart. Low concentration of divalent cations leads to a segregation of photosystems [20]. According to surface charge theory the segregation of PS I and PS II is a cooperative effect of lipid and proteins which creates regions of low surface charge in which proteins contribute to the increase in van der Waals' attraction [21,22]. High concentration of divalent cations leads to stacking, especially abundant in regions containing PS II. It is accepted that stacking results from interplay between van der Waals' attraction and electrostatic repulsion [21]. Wollman and Diner [23] showed that stacking of thylakoids and segregation of photosystems are two independent phenomena caused by two different ion-dependent mechanisms. According to them for monovalent cations the course of stacking is dominated by the segregation mechanism, and for divalent cations by the stacking mechanism. A decreased electrostatic repulsion due to a better shielding of the surface charges by a higher ionic strength causes shortening of the distances between membranes. This in turn brings the diffuse layers closer together [24]. A major function of the thylakoid is the development and maintenance of a transmembrane proton gradient of approximately three pH units. During photosynthesis the outer pH is typically near 8 and the inner is typically near 6, giving a pH difference of 2 across that is equivalent to 120 mV [25].

The present study was based on the idea that the inorganic ions (Na^+, Mg^{2+}) may modulate the activity of photosynthetic membrane systems in two nonspecific ways—via electrostatic field distribution at the membrane boundaries and due to their effect on the surface area/volume ratio, and therefore by changes in the particle geometry. We focussed on the direct contribution of the electrostatics on the thylakoid membrane light scattering (LS) properties. The investigation of the electrostatic contribution to the membrane ionic-exchange processes was another step towards the understanding of interaction between small peptide with thylakoid membranes, which are negatively charged as most of natural membranes.

The task of our study was to clarify the effect of Mt on electrophoretic mobility (EPM) of thylakoids like a unique model of biological systems as well as on their electron-transport processes at 'low-salt' and 'high-salt' medium under neutral conditions (pH 7.8). In that way we modulated different geometries (oblate and prolate thylakoid shapes) using a tiny difference in ionic strength of suspending medium. We studied the energization-induced changes in LS of thylakoids upon Mt treatment at both ionic strengths under saturating

light. The effect of Mt on the PS II effectiveness in the primary photochemical reactions was estimated by the parameters of prompt chlorophyll (Chl) fluorescence [26,27].

2. Materials and methods

2.1. Materials

Chemicals were of analytical grade. Mt was purchased from Sigma (St. Louis, MO, M-7391, HPLC) without further purification. Hepes (*N*-(2-hydroxiethyl) piperazine-*N*′-(2-ethanesulfonic acid) and NaCl were purchased from Merck. Glycerol and phenazine methosulfate (PMS) were obtained from Serva. $MgCl_2 \cdot 6H_2O$ and D-sorbitol were obtained from ICN Biochemicals and KH_2PO_4—from LOBA Chemie.

2.2. Preparation of thylakoids

Chloroplasts were isolated from the leaves of 2-week-old seedlings (*Pisum sativum* L., cv. Ran 1) grown in a greenhouse in a full nutrient medium.

Pea leaves were blended in a medium containing 67 mM Na_2HPO_4/KH_2PO_4 buffer, pH 7.8, 330 mM sorbitol, 5 mM $MgCl_2$ at 0 °C. The resulting slurry was filtered through eight layers of muslin. The filtrate was then centrifuged in a K23 'Janetzki', Leipzig bench top centrifuge at $200 \times g$ for 60 s. After centrifugation of the supernatant at $1000 \times g$ for 7 min the remaining pellet was washed twice in the same buffer. The final pellet was resuspended in the upper buffer to a concentration of 2 mg Chl ml^{-1} [28]. The thylakoid suspension was mixed with 10% glycerol (v/v) as a cryoprotectant and kept in liquid nitrogen until use [29].

For LS and zeta potential measurements the following buffers were used:

- 10 mM NaCl, 25 mM Hepes (KOH), pH 7.8;
- 5 mM $MgCl_2$, 25 mM Hepes (KOH), pH 7.8.

The thylakoid particles formed in this way had a size of 3–15 μm. The appropriate concentration of Mt was added to the solution before the incubation of the thylakoids. Thylakoids (final concentration of 6 μg Chl ml^{-1}) were diluted in 20 ml of appropriate buffer without or in the presence of following concentration ranges: 0.19–3.90 pM Mt; 19–390 pM Mt; 1.9–39 nM Mt; 0.03–1.75 μM Mt. Higher concentrations of Mt induced strong aggregation of the particles. The experiments were implemented at 25 °C after 5-min dark incubation of thylakoids with Mt added to a suspending medium. The stock solution of Mt (M_w 2847) was prepared in a mg Mt ml^{-1} of doubled distilled water (3.5×10^{-4} M).

2.3. Light scattering measurements

Experiments were conducted with a laboratory-made apparatus using the Specol 10 Spectrophotometer (Carl Zeiss, Jena). The ionic-exchange processes of LS were monitored at 550 nm as a function of micromole concentration of Mt (0.03–1.75 μM) in the presence of 40 μM PMS (a mediator of photosynthetic electron transport processes). Higher doses of Mt induced an opalescence of thylakoid suspension.

The scattering level at an angle of 90° represented the degree of aggregation. Cut-off filters were used to protect the photocell from the actinic light ($\lambda \geqslant 640$ nm) with saturating intensity of 4750 μmol m^{-2} s^{-1} at the position of the cuvette. A slide projector provided white light. Thylakoid membranes (6 μg Chl ml^{-1}) were added to the reaction medium (2 ml) and were incubated at temperature of 25 °C for 5 min.

Data were averaged of triplicate measurements.

2.4. Microelectrophoresis measurements

EPMs were measured using a cytopherometer (OPTON, Feintechnik Ges, m.b.H., Wien, Austria) using a rectangular cell and platinum electrodes. Electrophoretic migration of 15–20 particles was timed for both forward and backward (reversed field) runs over a known distance (32 μm). The standard errors of the EPM *u* were 2–8%. The effect of the peptide Mt on the EPM of thylakoids was measured after 5-min incubation at 25 °C and a Chl content of 6 μg ml^{-1}. The electrical conductance and viscosity of the different media, including the thylakoids, were measured using a Radelkis (Hungary) model, OK-104 conductome-

ter and a Rheo (Germany) viscometer, respectively.

The zeta potential, ζ, was calculated from the EPM, u, using Helmholtz–Smoluchowski equation:

$$\zeta = \frac{\eta \times u}{\varepsilon_r \times \varepsilon_0}, \quad (1)$$

where ε_r is the dielectric constant of the aqueous phase ($\varepsilon_r = 78.5$ at 25 °C), ε_0 is the permittivity of free space ($\varepsilon_0 = 8.8542 \times 10^{-12}$ F m^{-1}), η is the viscosity of the aqueous phase ($\eta = 0.0009$ Pa s, 25 mM Hepes, 10 mM NaCl, pH 7.8; $\eta =$ 0.0010 Pa s, 25 mM Hepes, 5 mM $MgCl_2$, pH 7.8) and u is expressed in value $u \times 10^{-8}$ m^2 V^{-1} s^{-1}.

When the thylakoid membrane surface is bathed in a medium containing a Z–Z electrolyte such as NaCl, the surface charge density (σ)

$$\sigma = 0.1174(C_{i\alpha})^{1/2} \sinh\left(\frac{Z\psi_0}{51.7}\right) \quad (2)$$

at 25 °C, where σ is in C m^{-2}, $C_{i\alpha}$ is in mol l^{-1} and ($\psi_0 \approx \zeta$) is in mV [22].

2.5. Chlorophyll fluorescence measurements

Chlorophyll fluorescence emission was measured by a pulse amplitude modulated fluorometer (PAM 101-103, Heinz Walz, Effeltrich, Germany). The initial fluorescence yield in weak modulated light (0.075 μmol m^{-2} s^{-1} PPFD), F_0, and maximum total fluorescence yield emitted during a saturating white light pulse (1 s, over 3500 μmol m^{-2} s^{-1} PPFD, by Schott KL 1500 light source), F_m, were determined. The Chl concentration was 50 μg ml^{-1} and the sample volume was 1 ml. The samples were diluted in the respective buffers in the presence of following concentrations: 0.17–3.50 pM Mt; 0.017–0.350 nM Mt; 2–33 μM Mt.

The data presented are means of three different experiments (or chloroplasts + isolations), each including at least three replications. Experimental data were processed statistically by the Student's t-test.

3. Results

3.1. Effects of melittin on the EPM of thylakoids

Fig. 1 shows the influence of Mt on the electrokinetic properties of thylakoids at 'low-salt' ($I =$ 0.01, closed circles) and 'high-salt' ($I = 0.015$, closed triangles) medium. Changing the ion content of the medium (i.e. in the presence of 5 mM Mg^{2+} cations) induced a rapid change in volume of thylakoids, which was accompanied by alteration in LS.

In the case of 'low-salt' thylakoids, Mt induced a significant change in EPM of pea thylakoids at picomole (Fig. 1A and B) and nanomole concentrations of treatment (Fig. 1C). The results showed an increase in EPM with increasing Mt concentration. There was an electrostatic effect of Mt-induced transition from swelled thylakoids to structured aggregates due to a change in structure determinating properties of the membrane at picomole and nanomole doses. However, when thylakoids ($I = 0.01$) had been pretreated by 1–1.9 pM Mt (Fig. 1A), 146 pM Mt (Fig. 1B), 9.7–14.5 nM Mt (Fig. 1C), a considerable EPM drop (> 14%) was measured. The transition from swelling particles to appressed thylakoids with a less effective surface for Mt molecules changed the interaction of Mt with outer surface of unstacked membranes. This change in geometry of the particles caused a decrease in the level of Mt binding to hydrophobic parts of membrane involved in appropriate peptide-membrane interaction. We specified these Mt concentrations as critical transitions from unstacked to stacked structures followed by swelling of aggregates at higher concentrations of the appropriate picomole and nanomole doses of Mt. When thylakoids were pretreated with doses of 0.09–0.35 μM Mt no change in EPM was measured in comparison to untreated thylakoids (Fig. 1D). Only addition of 0.035 μM Mt caused a 20% effect of increase in zeta potential of thylakoids (with ≈ 5 mV, i.e. from $\zeta = -25.6$ mV for untreated 'low-salt' thylakoids to $\zeta = -30.8$ mV in the presence of 0.035 μM Mt in suspending medium). Obviously, Mt increased the net negative surface charge density of 'low-salt' thylakoids (σ varied from −0.0061

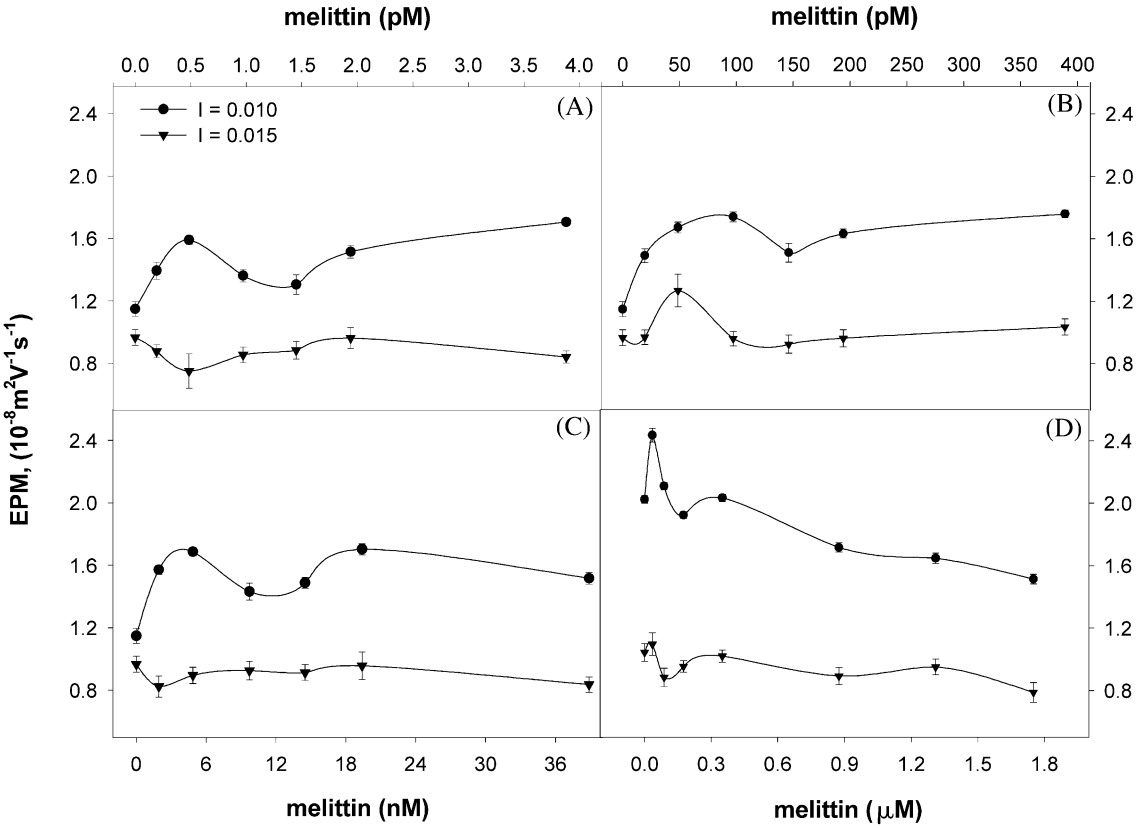

Fig. 1. Effect of Mt on EPM ($-u\times10^{-8}$, m^2 V^{-1} s^{-1}) of 'low-salt' (I=0.01) and 'high-salt' (I=0.015) pea thylakoid membranes. Mt dissolved in buffers of different ionic strength was mixed with thylakoids at 25 °C for 5 min. The buffers used were 25 mM Hepes (KOH), 10 mM NaCl, pH 7.8 and 25 mM Hepes (KOH), 5 mM $MgCl_2$, pH 7.8 and concentration of 6-μg Chl ml^{-1}.

C m^{-2} without Mt to -0.0074 C m^{-2} in the presence of 0.035 μM Mt) which were well structured like stacked complexes of thylakoid membranes due to an additional exposure of negative surface exposed groups on the surface of the membrane. The strong decrease by 15–25.5% in EPM of unstacked thylakoids was determined for 0.87–1.75 μM Mt used in the suspending medium at pH 7.8 (Fig. 1D). The EPM values varied from -2.02×10^{-8} m^2 V^{-1} s^{-1} (in the absence of Mt) to -1.30×10^{-8} m^2 V^{-1} s^{-1} at final concentration of 2.65 μM Mt (data not shown). It was accompanied by a process of swelling of aggregate complexes upon 1.3–2.65 μmol doses of peptide molecules. The influence of Mt binding (micromole concentrations) on thylakoids confirmed the specific bee venom peptide destabilization of membrane surface and reduction of the electrostatic charge at the surface due to build-up of positive surface potential in the presence of Mt and decreased proton concentration at the interface [30]. The micromole Mt-induced decrease of net negative surface charge density of 'low-salt' thylakoids was a result of dehydration-dependent conformational rearrangement of thylakoid membrane components. The influence of Mt modification of negative charges on the thylakoid membranes could facilitate the entropy-driven 'depletion–attraction' between the adjacent membranes [18]. The picomole and nanomole concentration range of Mt induced the change in geometry of the particles and swelling of stacked structures

as well as different permeability properties of their thylakoid membranes at the lower ionic strength. The increased net negative surface charge density of the particles was due to the exposure of additional negatively charged groups on the outer surface of thylakoid membranes upon polyvalent molecules.

The electrostatic effect of Mt molecules on thylakoids in the presence of Mg^{2+} cations was slightly expressed (Fig. 1, closed triangles). Only doses of 48 pM Mt induced a large increase with 31% in EPM of 'high-salt' thylakoids (Fig. 1B). The EPM increased from -1.00×10^{-8} $m^2\,V^{-1}\,s^{-1}$ in the absence of Mt to -1.27×10^{-8} $m^2\,V^{-1}\,s^{-1}$ after Mt treatment. The zeta potential increased by 5 mV from −13.9 to −18.2 mV, respectively, which correspond to an increase in surface charge density of 'high-salt' thylakoids from −0.0047 to −0.0064 $C\,m^{-2}$ in the presence of 48 pM Mt. This effect was due to specific interaction of Mt with the photosynthetic membrane components accompanied by a change in the permeability properties of the thylakoids and an appropriate change from swelling thylakoid to stacking complexes. We expected the lower surface area/volume ratio of 'high-salt' thylakoids the less pronounced binding efficiency of Mt in comparison with a 'low-salt' thylakoids. It could explain the insignificant change in Mt-induced EPM effect on thylakoid membrane at higher ionic strength ($I=0.015$). The EPM of thylakoids in the presence of 0.48 pM Mt (Fig. 1A) or 1.75 μM Mt (Fig. 1D) were reduced with approximately 20%. Mt molecules decreased the EPM of stacked thylakoids from -1.04×10^{-8} $m^2\,V^{-1}\,s^{-1}$ without peptide to -0.79×10^{-8} $m^2\,V^{-1}\,s^{-1}$ in the presence of 1.75 μM Mt in suspending medium. This meant a decrease in zeta potential from −15.0 to −11.3 mV, respectively, and appropriate inhibition of net negative surface charge density from $\sigma=-0.0051$ $C\,m^{-2}$ to $\sigma=-0.0038$ $C\,m^{-2}$ after the same highest micromole Mt doses.

The higher micromole doses of Mt induced a decrease in the surface electrical properties of stacked thylakoids because of stronger electrostatic interactions with the membrane due to higher net negative surface charge density of the particles. Besides the optimal level of the appression of the adjacent regions between the membranes were the prerequisite for the less surface area expressed for binding of Mt with negatively charged surface groups at the margins of the thylakoid complexes. There was a compensation of the negatively charged surfaces of thylakoids and after lower doses of Mt the swelling of particles was observed (Fig. 1A–C). On the contrary, the highest Mt concentrations were not able to compensate the excess of negative charges on the surface of stacked 'high-salt' thylakoids instead of the aggregation of stacked thylakoid complexes.

3.2. Effects of melittin on the light scattering of thylakoids

Light scattering dependence was observed in the amplitude and the kinetics of light-induced increase (which includes two main phases—fast phase and slow phase) of the LS of thylakoid suspension. We used the term 'basal LS' to describe the stacking level of photosynthetic membranes. The intensity of photoinduced scattering exponentially decreased after turning off the actinic light, but did not always reach the initial state. Later, we used the term decay phase of LS describing the dark relaxation of the system. The LS response of the thylakoid membrane at actinic light was shown schematically in Refs. [31,32]. The kinetic phases depended on the cation concentration in the medium. During the fast phase the ion effect is realized through modification of the double electrical layer on the thylakoid membrane surface and in the slow phase through their participation as counterions in the formation of ΔpH. After a period of fast ionic exchange processes the system reached a steady state LS_{max}. We described the LS response in terms of the three main parameters LS_{min} (fast phase), $LS_{max-min}$ (slow phase) and $LS_{max-stat}$ (decay phase). Continuous illumination leads to a net bulk proton influx of magnitude depending on the difference between turnovers of proton influx and efflux [33]. Under continuous illumination of thylakoid membranes, alteration of the membrane potential reflected in the fast phase of LS. If the proton pump is electrogenic, a membrane potential is established, leading to accumulation of protons and a change

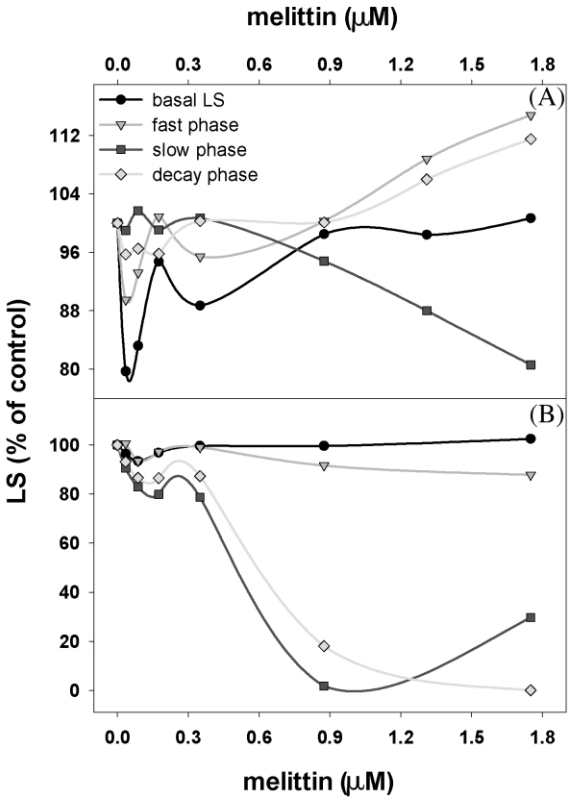

Fig. 2. Effect of Mt on the LS of pea thylakoids suspended at different ionic strengths. Mt was mixed with thylakoids (6-μg Chl ml^{-1}) at 25 °C for 5 min. The buffers were 25 mM Hepes (KOH), 10 mM NaCl, pH 7.8 (A) and 25 mM Hepes (KOH), 5 mM $MgCl_2$, pH 7.8 (B).

in pH [34]. Thus, the energy-dependent proton translocation is related to the secondary ion exchange processes. Consequently, the slow phase of LS represented a transmembrane proton gradient formation. When the light was turned off, the ionic gradients equilibrate as the influx of protons stops immediately [35].

A maximal increase of the fast phase of LS of 'low-salt' thylakoids (Fig. 2A) at highest doses of 1.3 and 1.75 μM Mt was shown. The similar enhancement in relaxation ability of the particles under Mt treatment was observed. On the contrary, the same concentrations of Mt caused an inhibition of ΔpH formation. The decrease in ΔpH presumably reflects a decreased contribution of cyclic electron transport around PS I. Mt decreasing proton conductivity leads to an increase of the electric potential towards the primary ionic-exchange processes flow.

Mellitin polyvalent cations did not significantly change the level of aggregation of stacked thylakoids in comparison to the basal LS of 'high-salt' thylakoids in the absence of Mt in suspending medium (Fig. 2B). The basal LS and fast phase of LS had similar course after Mt treatment of stacked thylakoids and did not change appreciatively compared to the control values without the peptide at pH 7.8.

The slow phase of LS of stacked thylakoids decreased rapidly with approximately 90% in the presence of 1.3–1.75 μM Mt concentration range. There were no changes in secondary ionic-exchange processes of 'high-salt' thylakoid membranes after lower doses of Mt treatment at pH 7.8 (Fig. 2B). The stronger decrease in the ΔpH formation in comparison to 'low-salt' thylakoids was observed. Hence, a stronger decrease contribution of cyclic electron transport around PS I might be explained by the Mg^{2+} efflux from the thylakoids after intensive illumination and increase of concentration of magnesium cations [36] that change the ionic environment, containing an appropriate concentration of $MgCl_2$, and altered the course of slow phase of LS. The inhibition of the secondary ionic-exchange processes after highest doses of Mt treatment was accompanied by a similar decrease in relaxation of 'high-salt' thylakoids after turning off the light. The decay phase decreased strongly with approximately 80% at the highest concentration range of 1.3–1.75 μM Mt. In this case permeability of other ions (like Mg^{2+}, K^+) did not dominate over the permeability of protons. Obviously, the efflux of protons was strongly inhibited due to the screening effect of Mg^{2+} or K^+ ions, translocated outwardly during illumination of Mt-treated thylakoids in the presence of 5 mM $MgCl_2$ in suspending medium.

Effect of Mt on the primary photochemical activity of PS II of thylakoids was also investigated. The results obtained showed that the primary photochemical activity of PS II, estimated by the ratio of F_v/F_m, was not influenced by the low concentrations of the peptide Mt (Fig. 3). The primary photochemical activity decreased only

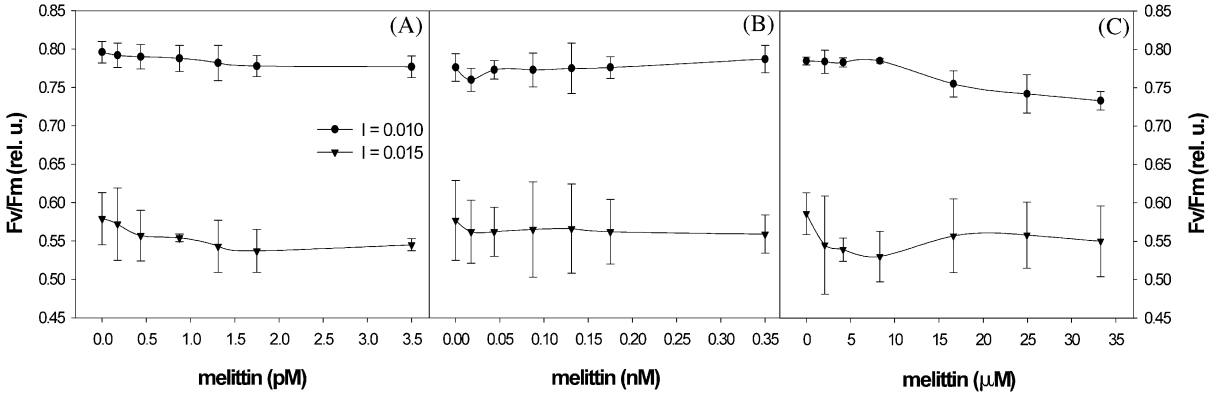

Fig. 3. Effect of Mt on the F_v/F_m ratio of 'low-salt' and 'high-salt' thylakoids. Suspending media consisted of 25 mM Hepes (KOH), 10 mM NaCl, pH 7.8 ($I=0.01$) and 25 mM Hepes (KOH), 5 mM $MgCl_2$, pH 7.8 ($I=0.015$) and Chl concentration of 50 μg ml^{-1}.

when chloroplasts were incubated with higher Mt concentrations and this effect was better expressed in 'high-salt' stacked than in 'low-salt' unstacked thylakoid membranes.

4. Discussion

Mellitin molecules can affect the bilayer structure of biological membranes at very low concentration [37]. They can first stick into the lipid bilayers and loosen the lipid arrangement of the membrane. At neutral pH thylakoid membranes carry a surplus of negative charges on their outer surface [1,3]. These surface charges are mainly due to the carboxyl groups of glutamic and aspartic acid residues of exposed portions of integral proteins. Negatively charged polar lipids such as phosphatidylglycerol or sulfolipid could also be responsible for the negative surface charges of intact chloroplasts [8,19,20]. The intervention of the Mt molecules with their two effective positive charges at pH of 7.8 [8] must significantly affect the surface potential and the distribution of surface charges and therefore may cause the change in the protein conformational environment.

Mellitin strongly increased the EPM of 'low-salt' thylakoids ($I=0.01$) at picomole and nanomole concentration range but inhibited it at micromole concentrations. Mt did not effectively change the EPM of 'high-salt' thylakoids ($I=0.015$). In experiments using 'low-salt' thylakoids, Mt increased the primary electron transport processes across 'low-salt' thylakoids, while it did not affect that of 'high-salt' thylakoids. The latter result related closely to the fact that this peptide acted strongly on the generation of electrical potential difference ($\Delta\psi$) across the thylakoid membrane at lower ionic strength. The increase obtained in the maximum level of the primary electron transport processes of 'low-salt' thylakoids at pH 7.8 upon Mt treatment (1.75 μM) was presumably due to the collapse of ΔpH by proton efflux through the coupling factor [30]. The same level of large enhancement of dark relaxation processes through the 'low-salt' thylakoid membranes accompanied by an increase in proton efflux or rapid migration to the bulk phase was observed. The primary ionic-exchange processes may be coupled with the level of aggregation between the particles at lower ionic strength ($I=0.01$), and both with the relaxation processes after turning off the light were more likely to be activated by the changes caused by Mt at micromole concentration range. A 5 mM $MgCl_2$ solution caused screening of negative charges on the stacked membranes and Mt did not affect the electric potential between 'high-salt' thylakoids. The lack of significant change in surface charge density on the particles at higher ionic strength was observed. Mt (micromole concentration range) decreased the

proton gradient generation on the membrane at both ionic strengths, but it affected more strongly the 'high-salt' than that of 'low-salt' thylakoids. These results indicated that Mt penetrated into the membranes and the adsorption on the surface was affected by electrostatic interactions.

The cross-membrane proton gradient (ΔpH) of stacked thylakoids was inhibited at higher concentration of Mt due to delocalization of the pumped protons. Mt did not alter electrokinetic potential of stacked thylakoid membranes but exchanging with co-ions of pumped protons was jeopardized by the presence of immobile peptide adjacent to the membrane. The effect of peptide on thylakoids at high level of stacking was expected to inhibit the possibility of the photosynthetic membranes to reach the preliminary level of proton migration by membrane energization because of the screening by cations impeding the interaction of Mt with outer membrane surface groups.

In our previous experiments [9], we found that the nonionic detergent Triton X-100 could also increase the primary and decrease the secondary ionic-exchange processes, but it did not cause rapid enhancement in the level of aggregation of thylakoid membranes and proton release after turning off the light. Because Triton X-100 could also destroy lipid bilayers but did not act with membrane protein directly [38], we suggested that this difference was caused by one kind of direct electrostatic interaction between protein components and Mt. The interaction between Mt (higher concentration range) and thylakoid suspension ($I=0.01$) was also a result of protein aggregation and was caused by the decrease in surface charge density but not by the collapse of lipid environment. The destruction of lipid bilayers would also increase the aggregation of membrane proteins and hence, the increase in basal level of LS was observed at higher doses of Mt in comparison to its lower values at the lowest micromole concentration of Mt. Upon Mt treatment at higher doses, there was a recovery of aggregate ability up to its initial value of unstacked and untreated thylakoid membranes. The light-induced changes in LS from energized thylakoids correlated with ultrastructural changes [23,9,36]. The insertion of Mt molecules into thylakoid membranes disturbed the lipid arrangement and raised the net negative surface charge density at low-salt medium. Higher concentrations of Mt decreased EPM, zeta potential and surface charge density of thylakoids due to electrostatic interactions with the negatively charged surface exposed groups on the protein components. The electrostatic interaction between Mt and thylakoid membrane caused inhibition of the ΔpH gradient formation at $I=0.01$, but increase in dark relaxation of the photosynthetic system because of Mt-mediated aggregate formation of unstacked thylakoids and due to uptake of magnesium cations in outer thylakoid bulk solution upon illumination. At 'high-salt' medium, the experiment showed that generation of ΔpH across the thylakoid membrane as well as the proton release after turning off the light were not accompanied by a change in surface charge density of stacked thylakoids in the presence of lower micromole concentrations of Mt treatment. In this case, the Mg^{2+} ions accumulated in outer thylakoid space and in bulk solution during light energization could interrupt the exchange of protons and by this way inhibit the secondary ionic-exchange processes. Hence, it was expected that Mt molecules would act with proteins directly and cause strong inhibition of the outwardly directed translocation of protons across the stacked thylakoid membrane after switching off the light.

5. Conclusions

The interaction of Mt with thylakoid membranes was strongly dependent on the geometry of the particle. Thus, although biological membranes have a complex, multicomponent nature, the study of complex models like thylakoids can give useful information on the mechanism of the peptide–membrane interactions. The mode of action involved the interaction of Mt with thylakoid membranes by altering some functional properties of these membranes.

We evaluated, through the LS kinetics of thylakoid particles in the presence of Mt, the primary and secondary ionic-exchange processes through the membranes under saturating light. The electrostatic interaction was demonstrated by microelectrophoresis, which showed that the shell of positive

charges formed by Mt around the bilayer surfaces was affected by the ionic strength at neutral conditions.

It was possible to correlate the behavior of Mt with its capability of causing aggregation of membranes: most likely each Mt molecule bound to phosphate groups only in the plane of lipid bilayer. An electrostatic interaction to the negatively charged protein groups exposed on the surface of thylakoid membrane was also occurred. This prevented thylakoids from structural changes.

Our results showed that such surface concentrations of Mt could affect the function of molecules involved in ion transport and hence the primary and secondary ionic-exchange processes through the photosynthetic membrane system. The Mt–membrane interaction changed the efflux of protons in thylakoid membranes and thus, controlled the geometry of these particles in modulating membrane function.

Mellitin-induced electrokinetic potential changes in thylakoid membrane caused particle aggregation of positively charged peptide, which exhibited different affinity for these thylakoids at low ionic-strength media. In addition, the interaction of Mt in lower ionic strength was shown to be largely reversible. The data clearly demonstrated that the electrical charge on the outer surface of thylakoid membrane was capable of greatly affecting membrane–peptide interactions.

This brought us back to one of the main motivations for studying the biophysical effects of Mt on biological membranes, which was related to the ongoing discussion of the possible mechanism of peptide–membrane interaction. We suggested that the results presented in this paper give support to the hypothesis that the effect of peptide may be specifically controlled by the tendency for induced dynamical changes in the geometry of the particles. The accumulation of the amphiphilic molecules granted the bee venom peptide molecules more direct access to membrane proteins. They also altered an appropriate level of electron-transport processes in thylakoids. Our results should therefore be of interest in relation to the current discussion of the influence of Mt on the thylakoid membrane biophysical characteristics.

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